Morphologic Changes Correlating to Different Sensitivities of *Escherichia coli* and *Enterococcus faecalis* to Nd:YAG Laser Irradiation Through Dentin

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Background and Objective: **Previous studies demonstrated the disinfecting potential of Nd:YAG laser irradiation on the root canal system from an overall quantitative viewpoint. The aim of this study was to evaluate the specific effect of irradiation through dentin on gram-negative and gram-positive bacteria with regard to their cell structure.**
Study Design/Materials and Methods: **Sterile dentin samples of standardized size were divided into two sets of four groups with eight samples each. The first set was inoculated with *Escherichia coli* as the gram-negative test strain, the second set was inoculated with *Enterococcus faecalis*, which served as the gram-positive test organism. The samples were then irradiated on the bacteria-free side in contact mode under constant scanning movement at an angle of 10° by use of the fiber optic of the Nd:YAG laser. Upon laser treatment they were critical point dried and subjected to SEM investigation. Another two sets of samples were prepared and irradiated in the same manner and evaluated by standard microbiological procedures to verify whether the observed morphologic alterations correlated to cell death.**
Results: **SEM investigations revealed damage pattens that increased with the amount of energy applied. Whereas the gram-negative test organism showed immediate structural injury, the gram-positive test organism required repeated application of irradiation. The microbiological examination showed reduction of both bacterial strains, yet to different extents.**
Conclusion: **Our study demonstrates the different morphologic impact of Nd:YAG laser irradiation through dentin on representatives of the two main groups of bacteria. It shows that the construction of the cell wall is crucial for their individual sensitivity to laser treatment. Lasers Surg. Med. 26:250–261, 2000. © 2000 Wiley-Liss, Inc.**

Key words: bacteria; cell damage; disinfection; endodontics; microbial colonization; teeth; scanning electron microscopy

INTRODUCTION

It is a well-known fact that a pulpal infection allows bacteria to invade the entire root canal system and that the three-dimensional tubular net-

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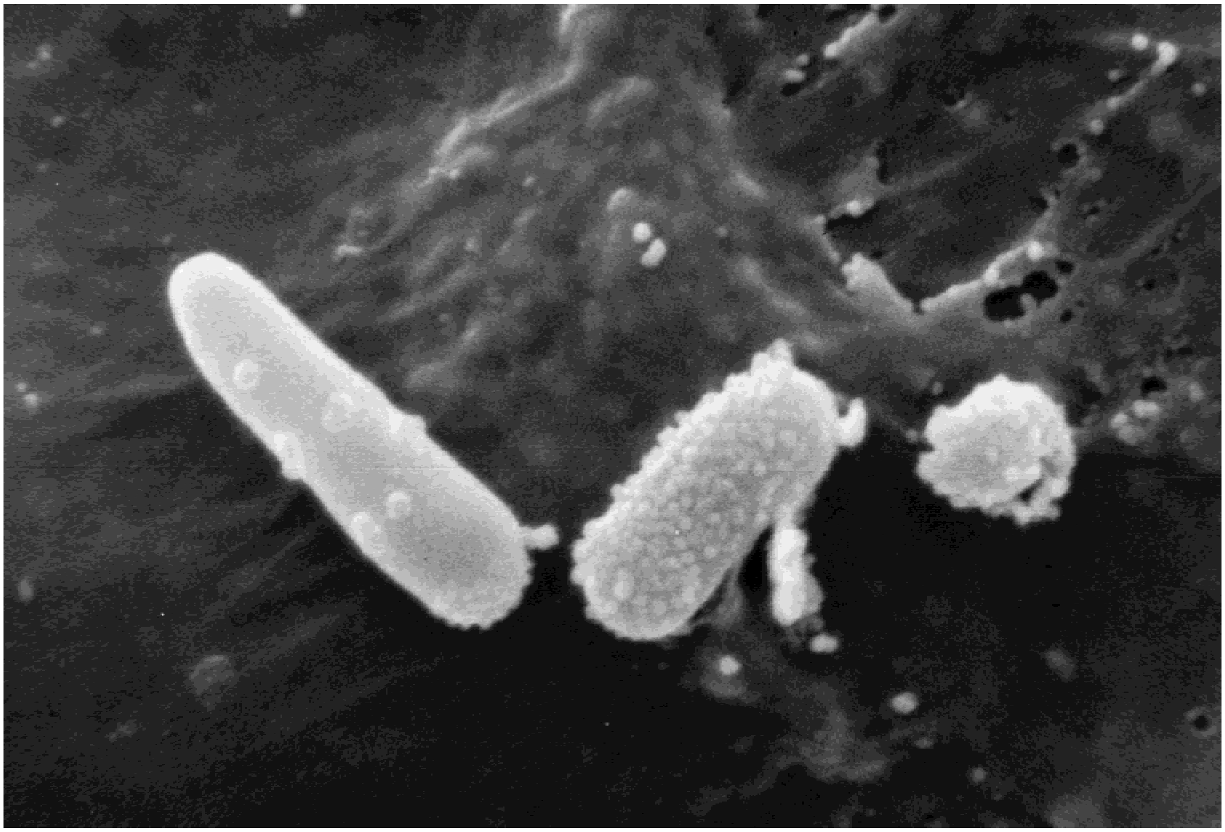

Fig. 1. Surface alterations in the form of protuberances like blebbing covering the cell bodies. Original magnification, 20,000×.

work provides a unique habitat where microorganisms can survive and multiply being out of reach of host defense mechanisms [1–3]. The microenvironment of the dentinal tubules favors the selection of relatively few bacterial types irrespective of the etiology of the infection process. Infections of the root canal system typically have a polymicrobial flora with approximately equal proportions of gram-negative and gram-positive bacteria [4,5]. These bacteria constitute an important reservoir from which root canal infection and reinfection may occur after endodontic treatment [6,7].

Conventional endodontic procedures aim to reduce the microbial load by rinsing the root canal with irrigants after preparation. Their germicidal potential is developed through direct cell contact as these chemical agents act by adsorbing to the cell envelope of microorganisms, thus breaching their functionality and causing leakage of metabolites and intracellular components [8]. Therefore, the fundamental condition upon which the disinfection of the dentin depends is the extent to which the chemical spreads into the dentinal tubules [9].

It has been demonstrated that a decrease in positive cultures after irrigation was only transient [10]. Furthermore, the penetration depth of irrigants has been recorded to be limited to a rather small area close to the root canal wall. Vahdaty et al. [11] stated that chlorhexidine reduced the bacterial counts only in the first 100 microns of dentinal tubules and that up to 50% of dentine samples remained infected. This finding is congruent with the observations of Berutti et al. [12] who reported the maximal penetration of irrigants to be 130 microns.

Experimental studies, on the other hand, have shown that bacterial strains are able to invade the tubules of radicular dentin to a much greater extent. The observations of Perez et al. [13] revealed that *Streptococcus sanguis* penetrated up to 792 microns within the tubules, whereas Kouchi et al. [14] showed that *Streptococcus mutans* can even invade the tubular system up to 1,150 microns. These findings provide conclusive evidence that the bacterial colonization established in the deeper layers of root canal dentin is virtually inaccessible to conventional irrigating procedures.

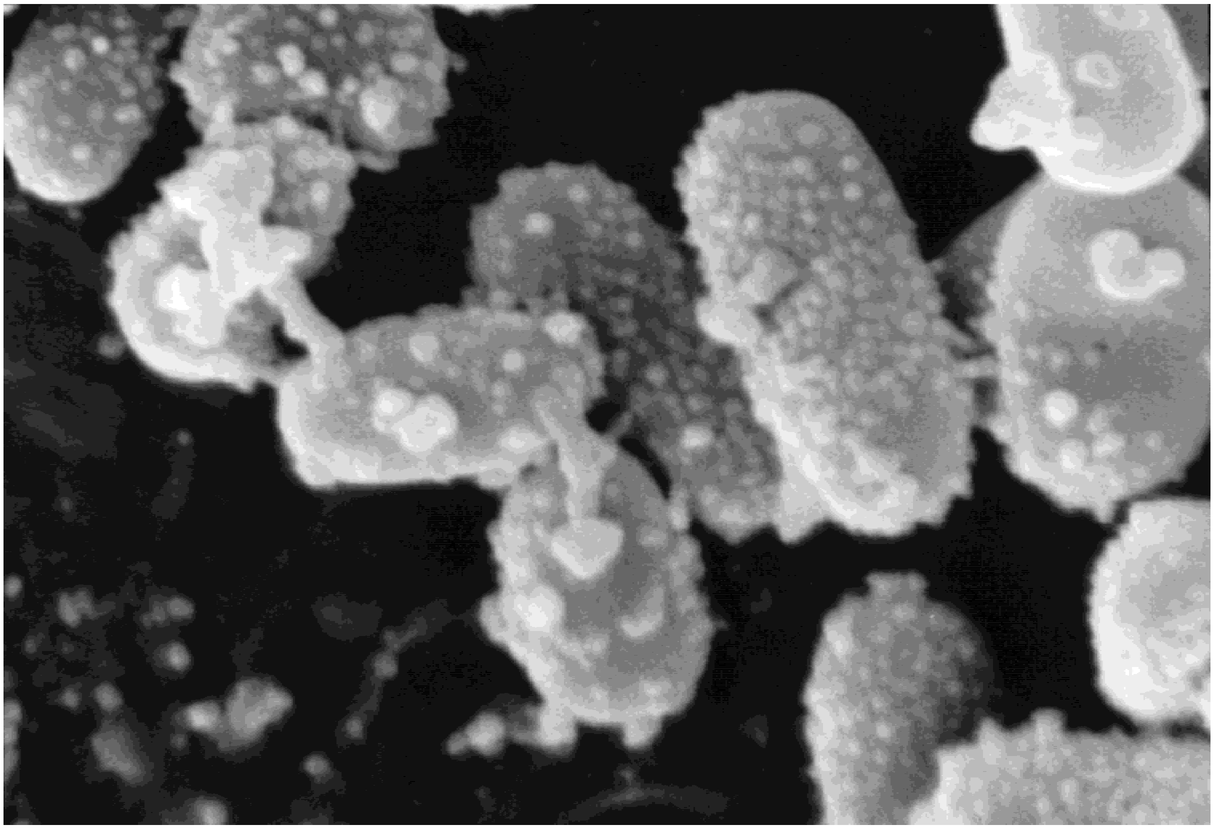

Fig. 2. Surface alterations in the form of irregular nodular structures of variable size covering a considerable number of the cell bodies. Original magnification, 30,000×.

Since the introduction of flexible glass fiber optics, the pulsed Nd:YAG laser emitting a wavelength of 1,064 nm has become a useful clinical device in successful modern endodontic therapy. Numerous authors have proved in vitro and in vivo that the Nd:YAG laser is a beneficial tool for disinfecting the root canal, its branches, and the adjacent dentin [15–17].

However, previous investigations focused on the superiority of the Nd:YAG laser in disinfecting the root canal and the dentin from an overall quantitative viewpoint, deducting their results exclusively from bacterial counts [18]. In addition to the established facts, the purpose of this study was to propose a more detailed approach to the selective dynamics of pulsed Nd:YAG laser irradiation on bacteria taking into account the specific architecture of gram-negative and gram-positive microorganisms.

We selected *E. coli* as our gram-negative test bacterium and *E. faecalis* as our gram-positive test bacterium for this study. These bacteria seemed particularly suitable because they are part of the microflora of the oral cavity [19–21], belong to the most thoroughly researched microorganisms, and yield reliable and reproducible results due to being easily cultivable.

Moreover, *E. faecalis* is of particular clinical relevance. This microorganism has been associated with therapy-resistant root canal infections [22–24]. This observation gains particular significance against the background of the limited effect of calcium hydroxide [25] and even high (6.5%) sodium hypochlorite concentrations [26] on this bacterium, as well as the bacterium's resistance against a steadily increasing number of modern antibiotics [27–29]. Once established in the dentin, it can barely be eradicated [24].

MATERIALS AND METHODS

Sample preparation

Noncarious, uniradicular extracted human teeth were selected and cut into 1-mm-thick plan-parallel longitudinal sections with a rotating diamond saw under continuos water irrigation according to the cutting technique by Donath (Cutting System, Exakt, Germany).

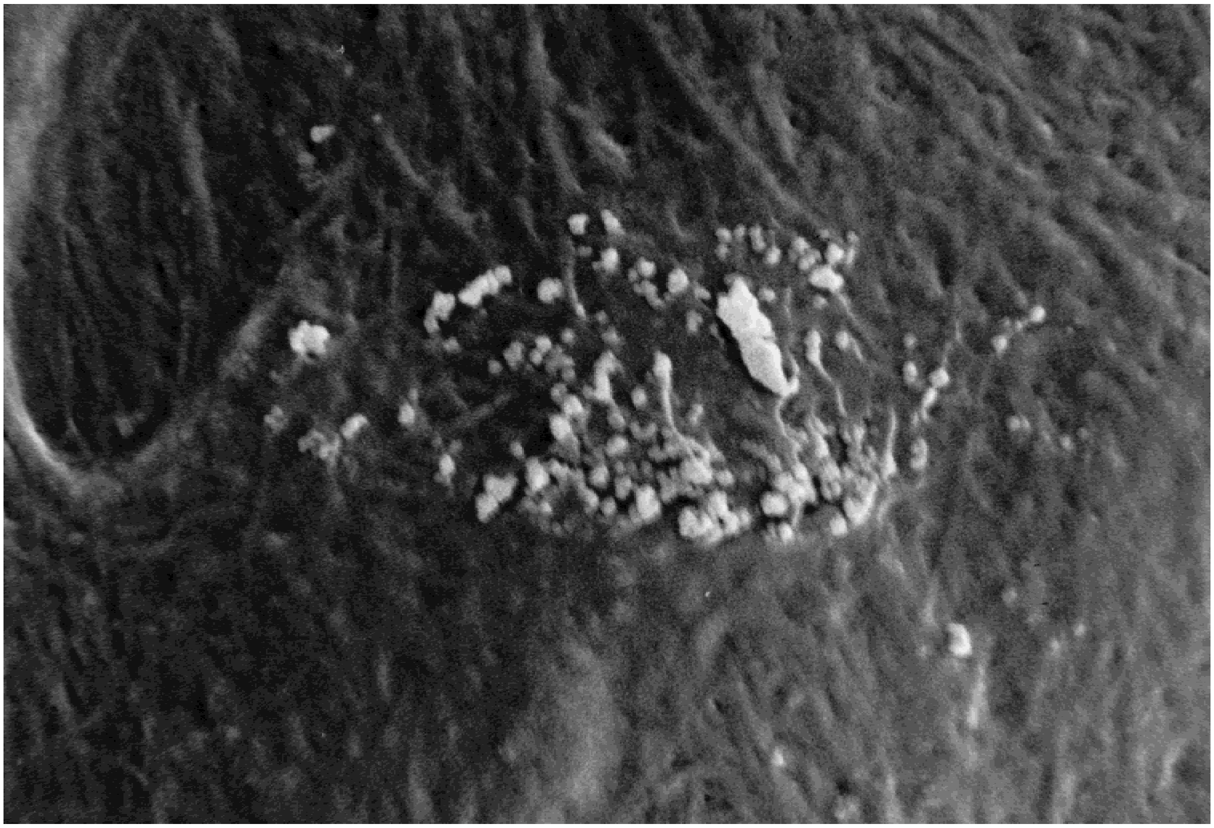

Fig. 3. For cells in group 2, the photomicrographs disclose completely disrupted cells surrounded by scattered cell fragments. Original magnification, 10,000×.

Rectangles measuring 2 × 6 mm were then cut from the upper and medium third of the dentin adjacent to the root canal. To ensure complete removal of the smear layer, the specimens were immersed in an ultrasonic bath with ethylenediaminetetraacetic acid for 4 minutes, followed by three washes with physiological saline solution for a period of 2 minutes each. Five specimens were selected at random and prepared for scanning electron microscopy (SEM) to confirm the effectiveness of this treatment. The samples were then stored in a 0.9% physiological saline solution and refrigerated at 4°C until use.

Laser Device

Laser irradiation was performed with a pulsed Nd:YAG laser, "Pulse Master 1000" (American Dental Technologies), emitting a wave length of 1,064 nm. The variable laser parameters are pulse energy (30–320 mJ) , pulse rate (10–200 pps), and power output (0.2–5.0 W). The pulse life is 100 μs. The laser beam is transferred to the handpiece by means of a flexible 300-micron fiber optic. The target beam is generated by a He/Ne laser (533 nm, 1 mW). To avoid deviations from the selected power setting, the output power at the end of the fiber optic was measured after every 5 seconds of irradiation by means of a powermeter (Lab Master Ultima/Coherent).

Temperature Measurements

Temperature measurements were performed to ensure that the experimental power settings used resulted in surface temperature rises of the dentin that did not exceed the range considered to be safe for in vivo endodontic application.

For this purpose, 20 dentin samples were divided into two groups. The first group was irradiated at 1.0 W, 15 pps, and the second was irradiated at 1.5 W, 15 pps. For each measurement, a dentin sample was placed on the platform of the TMG 1-9501 (a temperature measuring device designed by the Technical University of Vienna) and irradiated in contact mode under constant scanning movement at an angle of 10 degrees. Each lasing cycle comprised five irradiations of 5 seconds each with 15-second intervals.

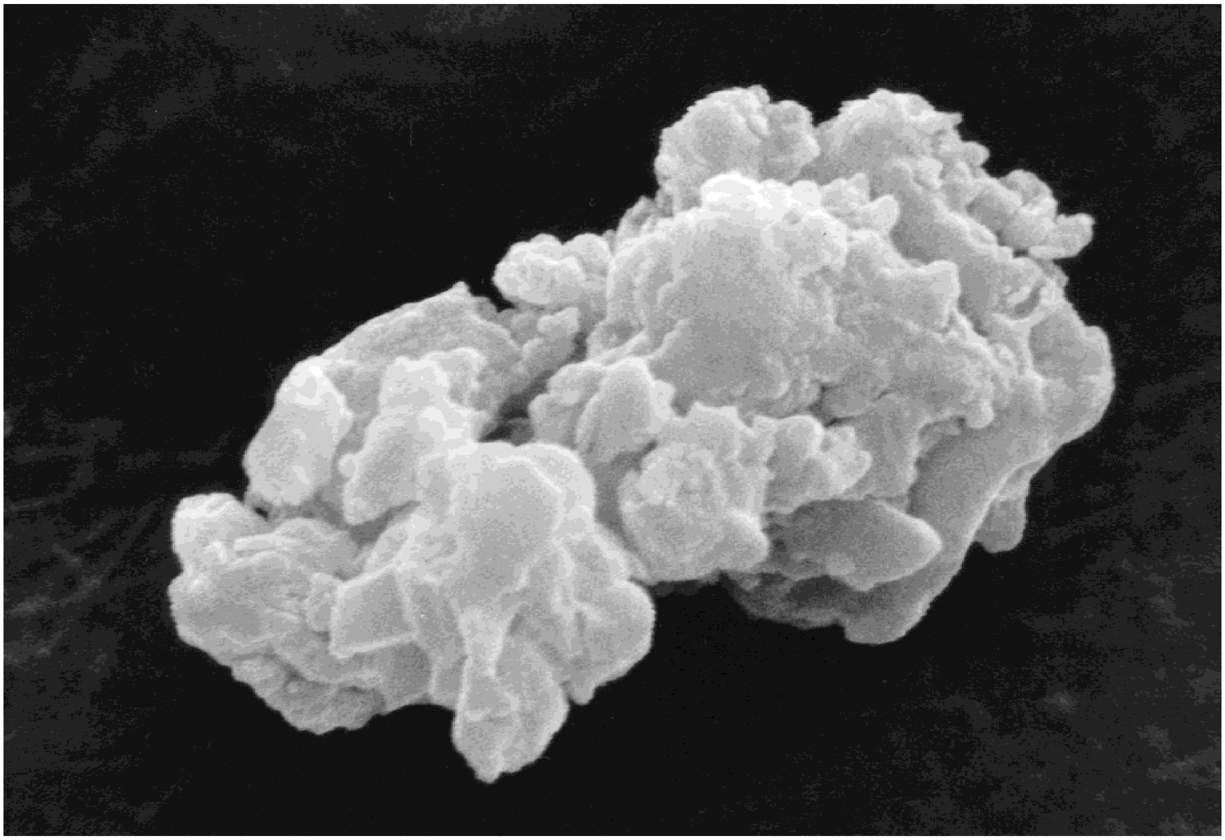

Fig. 4. For cells in group 2, coagulated, amorphous masses were seen, in which the shape of the bacteria merged with one another. The cell bodies seemed to be totally fused together. Original magnification, 13,000×.

Experimental Design

The dentin specimens were autoclaved for 10 minutes at 134°C to achieve sterility . They were then inoculated with 10 μl of either of the two reference strains, *Escherichia coli* (ATCC 25922) or *Enterococcus faecalis* (ATCC 29212) on one side, by means of a micropipette. The initial concentration of the suspensions was standardized at a cell density of 2×10^9 colony forming units/ml.

Two 10-μl portions of each stock solution, five dentin specimens inoculated with *E. coli* and five dentin specimens inoculated with *E. faecalis* were placed on culture plates and incubated for 24 hours; all of these served as viability control. This procedure ensured that the microorganisms were viable at the time of laser treatment.

SEM

The total number of specimens for SEM was 64; 32 were inoculated with *E. coli* and 32 with *E. faecalis*, respectively. Each group of 32 was then divided into four subgroups of eight samples each: control; 1.0 W-15 pps,1 cycle; 1.5 W-15 pps, 1 cycle; 1.5 W-15 pps, 2 cycles.

The following irradiation procedure was used: the specimens were irradiated on the bacteria-free side in contact mode under constant scanning movement of the optical fiber at an angle of 10 degrees. One lasing cycle comprised five irradiations of 5 seconds each, with 15-second intervals.

The specimens were then immersed in 2.5% glutaraldehyde at pH 7.4. After fixation, the samples were prepared for scanning electron microscopy investigation by dehydration in a series of graded ethyl alcohol solutions (50–100%) and acetone and were critical point dried [30,31]. Thereafter, the specimens were sputter coated and examined in a Jeol JSM-35 CF (Tokyo, Japan) scanning electron microscope with different magnifications at 15–25 kV.

Microbiological Examination

The total number of specimens for microbiology was 64. The samples were divided into the same subgroups and irradiated in exactly the same manner as for SEM evaluation.

Immediately upon irradiation, the speci-

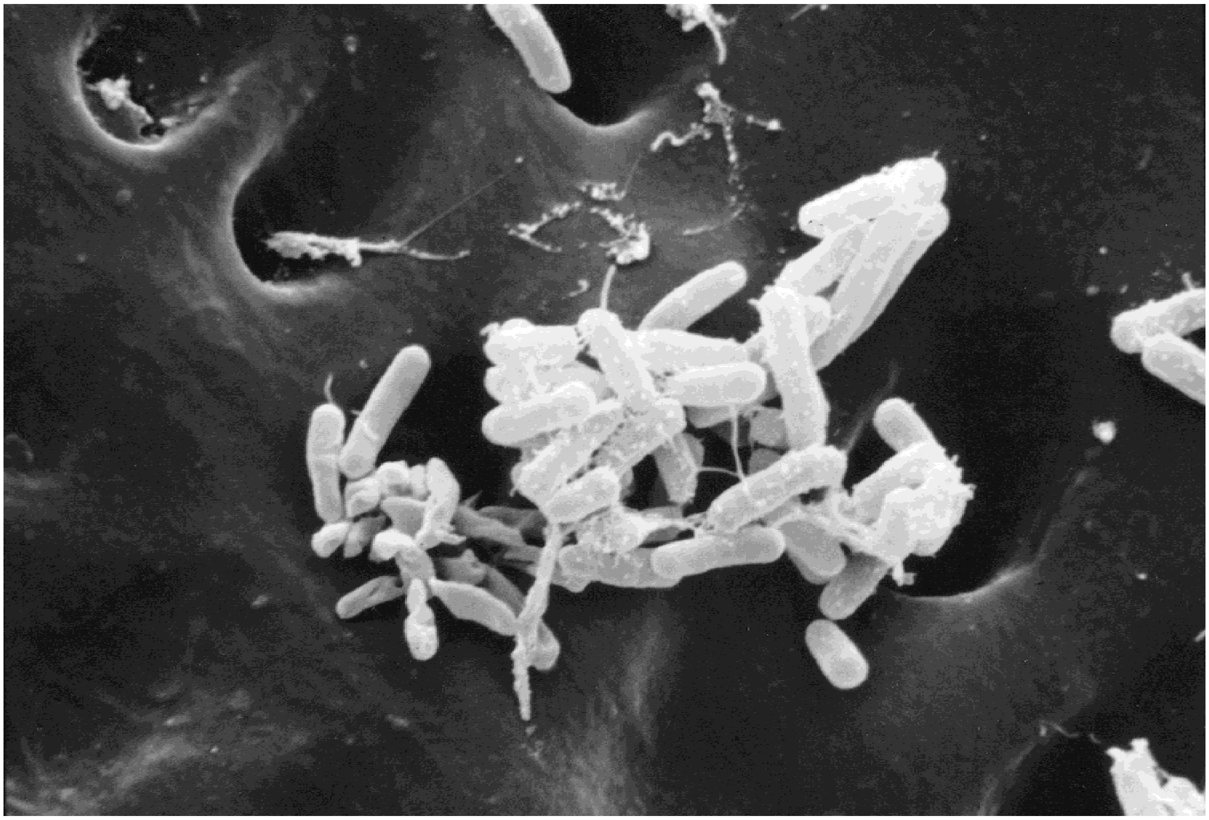

Fig. 5. Some group 2 bacteria appeared shriveled and empty, displaying perforations of their cell envelope. Original magnification, 6,000×.

mens were placed into sterile Eppendorf tubes and 250 μl of physiological saline solution were added. Each tube was then vortexed for 1 minute to remove the bacteria from the dentin and the tubules. Upon vortexing, 20 μl of the extracted fluid were diluted in log 10 steps. Fifty-microliter portions of the dilutions 10^{-1} to 10^{-7} were applied to the culture plates (sheep agar plates for *E. faecalis* and Mac Conkey for *E. coli*; Bio Mérieux, France) and incubated for 24 hours at 37°C. The colonies were then counted and the total number of bacteria (colony forming units per milliliter of the extracted fluid) was assessed.

RESULTS

Temperature Measurements

Temperature measurements revealed that irradiation at 1.0 W, 15 pps caused an average temperature rise of 3.8°C ± 0.1 on the dentin surface opposite to the irradiated side, whereas irradiation at 1.5 W, 15 pps resulted in an average temperature rise of 4.3°C ± 0.1.

SEM Investigations

SEM procedures provided clear evidence that the test organisms have different thresholds of sensitivity to irradiation and that the extent of radiation damage is directly proportional to the total amount of laser energy applied.

Group 1: *E. coli* irradiated at 1.0 W, 15 pps through 1-mm dentin. *E. coli* appears to be highly susceptible to irradiation. This power setting was sufficient to inflict severe membrane damage on this microorganism. A considerable number of cells showed surface alterations in terms of protuberances like blebbing and formation of irregular nodular structures of variable size covering the cell bodies (Figs. 1, 2).

Group 2: *E. coli* irradiated at 1.5 W, 15 pps through 1-mm dentin (1 cycle). At this setting, the photomicrographs disclosed completely disrupted cells surrounded by scattered cell fragments (Fig. 3). Furthermore, coagulated, amorphous masses were seen, in which the shape of the bacteria merged with one another. The cell bodies seemed to be totally fused together (Fig. 4).

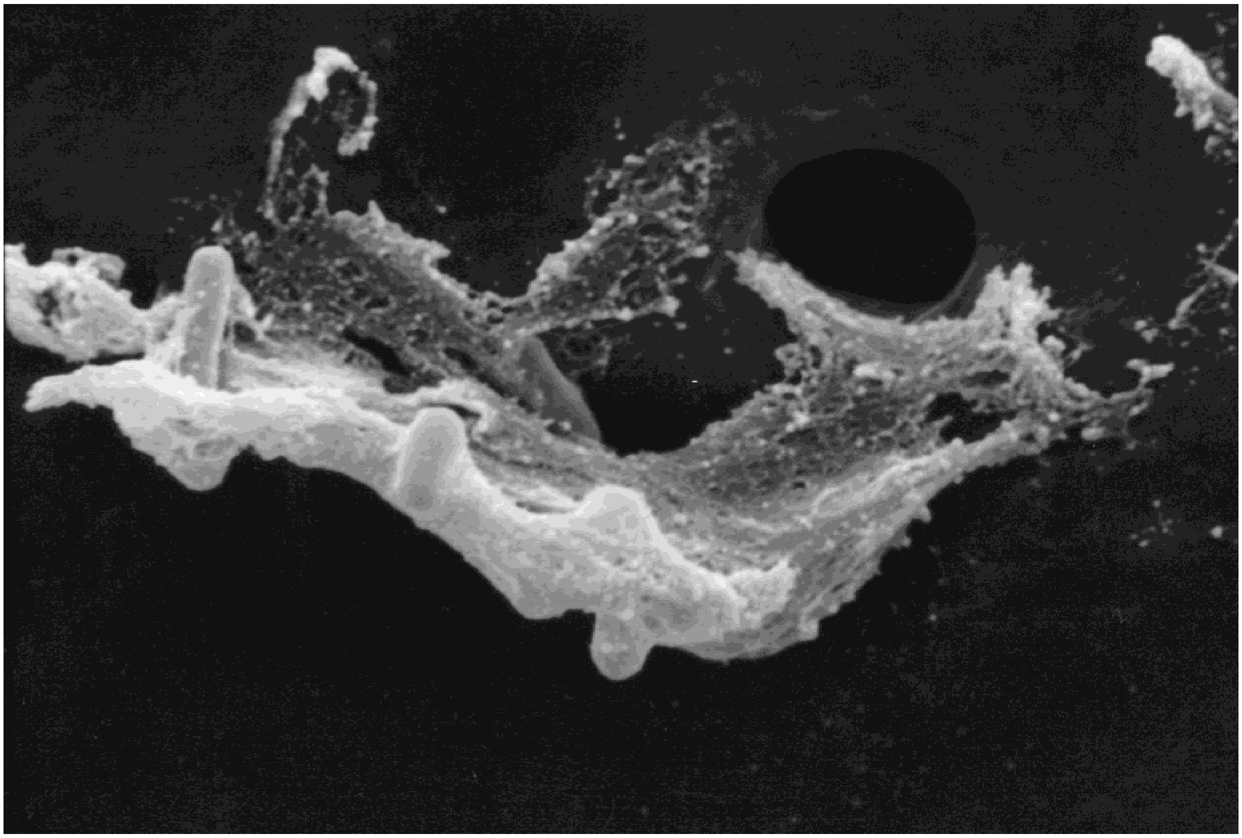

Fig. 6. Some group 2 did not exhibit degenerative morphologic features but were partly wrapped into bizarre candy-floss-like structures expanding between them. It is very likely that these structures are remnants of cell membranes. Original magnification, 7,800×.

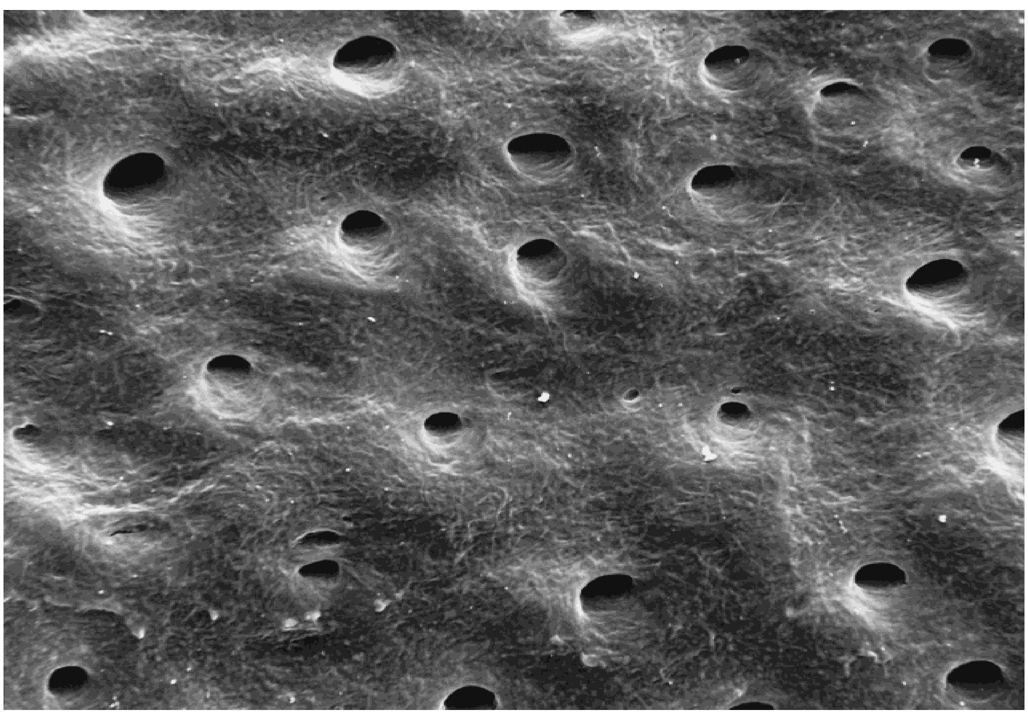

Fig. 7. For group 3, on the whole, the dentin surface appears uniformly clean. Only some cell fragments are visible. No morphologically intact bacteria are discernible. Original magnification, 2,400×.

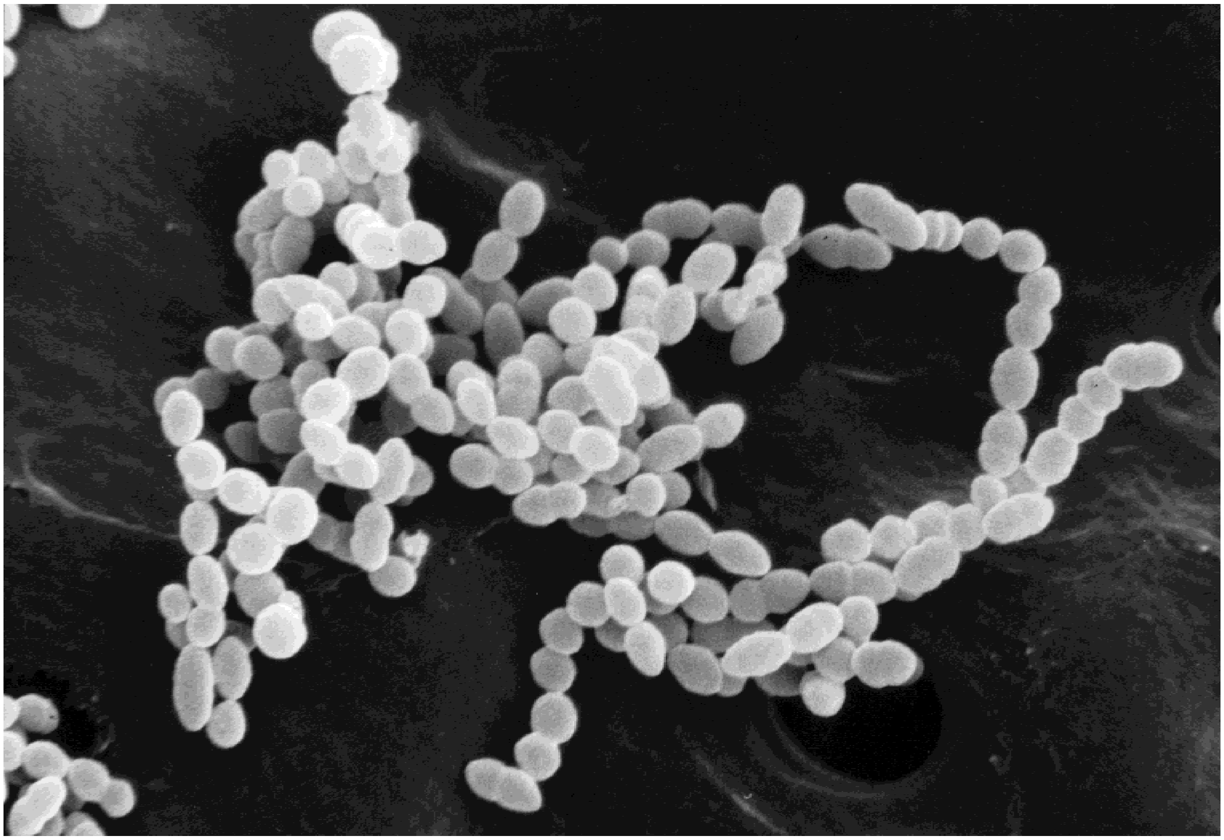

Fig. 8. The power setting used for group 4 was well tolerated by the gram-positive microorganisms. They mostly maintained their typical morphology without showing structural damage; yet some megalospheres (cells of increased size) can be seen. Original magnification, 10,000×.

Other bacteria appeared shriveled and empty, displaying perforations of their cell envelope (Fig. 5). Some cells did not exhibit degenerative morphologic features but were partly wrapped into bizarre candy-floss-like structures expanding between them. It is very likely that these structures are remnants of cell membranes (Fig. 6).

Group 3: ***E. coli*** **irradiated at 1.5W, 15 pps through 1-mm dentin (2 cycles).** On the whole, the dentin surface appears uniformly clean. Only some cell fragments are visible. No morphologically intact bacteria are discernible (Fig.7).

Group 4: ***E. faecalis*** **irradiated at 1.0 W, 15 pps through 1-mm dentin.** This power setting was well tolerated by the gram-positive microorganisms. They mostly maintained their typical morphology without showing structural damage; yet some megalospheres (cells of increased size) can be seen (Fig. 8).

Group 5: ***E. faecalis*** **irradiated at 1.5 W, 15 pps through 1-mm dentin (1 cycle).** This setting did not result in significant morphologic change either. The only noticeable feature was a variety in cell size and an increase of the number of megalospheres (Fig. 9).

Group 6: ***E. faecalis*** **irradiated at 1.5 W, 15 pps through 1-mm dentin (2 cycles).** After repeating the lasing cycle a second time, significant cell damage was evident. Completely disintegrated cells and scattered cell debris were found, as were morphologically unaltered cells in the immediate vicinity (Fig. 10).

Microbiological Evaluation

Effective bacterial reduction through laser irradiation. The average initial count of the *E. coli* control group was 1.7×10^7 (Table 1). The average count after irradiation at 1.0 W, 15 pps decreased to 4.3×10^5 corresponding to a logarithmic reduction of 1.6; after irradiation at 1.5 W, 15 pps the average count was 1.46×10^5, corresponding to a logarithmic reduction of 2.07. After two cycles at 1.5W, 15pps, the average count was 8.44×10^4, corresponding to a logarithmic reduction of 2.31. Expressed as a percentage, our

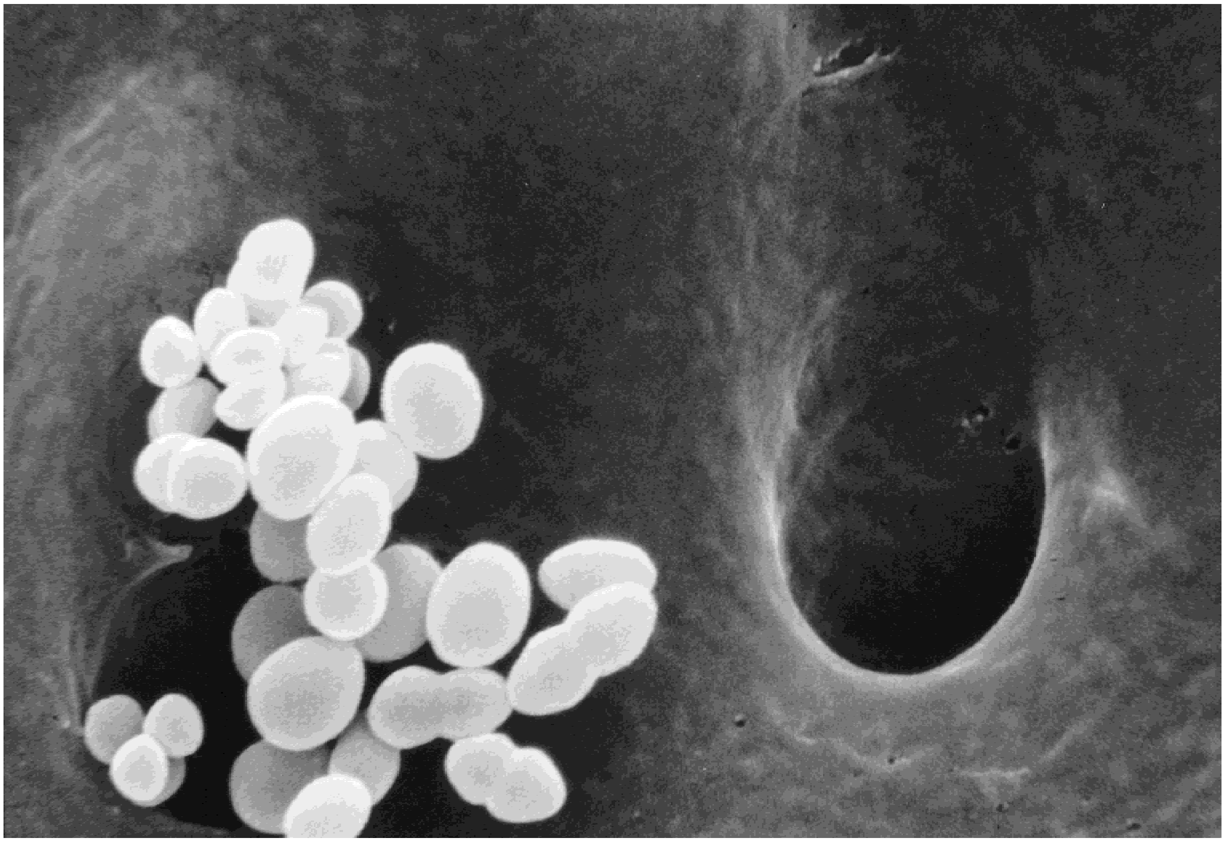

Fig. 9. The power setting used for group 5 did not result in significant morphologic change either. The only noticeable feature was a variety in cell size and an increase of the number of megalospheres. Original magnification, 7,200×.

findings show a bacterial reduction of 97.5% at 1.0 W, 15 pps, 99.2% at 1.5 W, 15 pps, and 99.5% at 1.5 W, 15 pps, and 2 cycles.

The average initial count of the *E. faecalis* control group was 4.31×10^7. The average count after irradiation at 1.0 W, 15 pps was 4.5×10^7, indicating an increase of 0.02 logs or 4.35%; after one cycle of irradiation at 1.5 W, 15 pps, the average count was still 3.39×10^7, corresponding to a logarithmic reduction of 0.10 and 21.5% of the bacteria killed. Bacterial reduction occurred after application of two lasing cycles at 1.5 W, 15 pps, which led to a decrease of the average count to 7.13×10^5. This corresponds to a mean logarithmic reduction of 1.78 and bacterial elimination of 98.4%.

DISCUSSION

The antimicrobial effect of laser irradiation in endodontic treatment has been stressed by numerous authors [15–18,32,33]. To date, only little information is available on the interaction between laser light and individual bacterial cells within the depths of the dentin layer. The question, thus, was whether there is a direct connection between the gram-specific cell structure and the bacterium's susceptibility to irradiation.

Our first test strain, *E. coli*, is a gram-negative rod possessing the typical cell envelope: its peptidoglycan or murein layer has a diameter of only about 2 nm, with only 15–20% of the cell wall being made up of it. The murein is intermittently cross-linked and surrounded by a complex outer membrane structure [34,35]. Lipoproteins, which constitute about 58% of the cell envelope, stabilize the outer membrane and anchor it to the murein. The lipopolysaccharides of the gram-negative cell envelope form part of the outer leaflet of the outer membrane [36].

E. faecalis, a spherical, gram-positive, highly resistant facultative anaerobe was chosen as second test strain. The main characteristic common to gram-positive bacteria as displayed by *E. faecalis* is the construction of their cell wall. Their murein is highly cross-linked, consisting of up to 40 layers, with a total diameter ranging from 20 to 80 nm. Also, 90% of the gram-positive cell wall

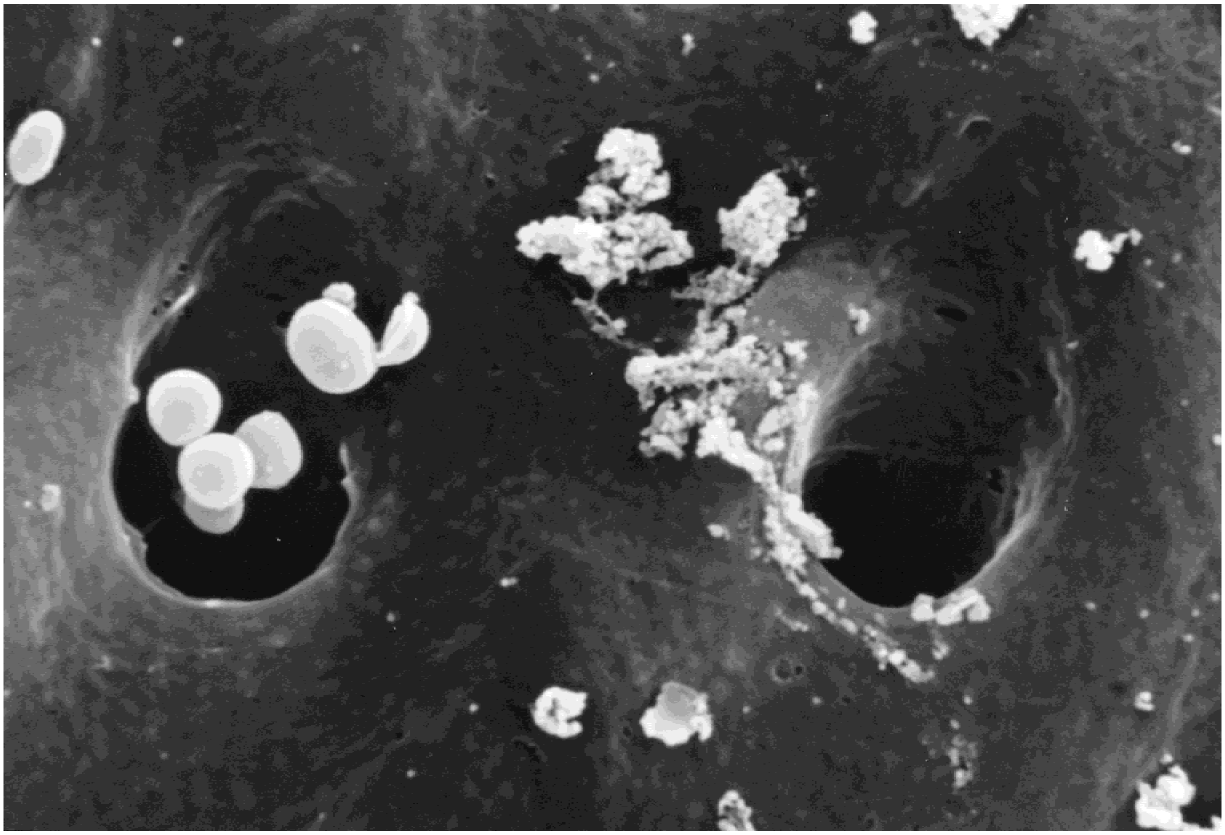

Fig. 10. In group 6, after repeating the lasing cycle a second time, completely disintegrated cells and scattered cell debris were found, as were morphologically unaltered cells in the immediate vicinity.

TABLE 1. Effective Bacterial Reduction Through Laser Irradiation

	Control group (untreated)	1.0 W, 15 pps, 1 cycle	1.5 W, 15 pps, 1 cycle	1.5 W, 15 pps, 2 cycles
Escherichia coli				
Mean bacterial count (CFU/ml)	1.72×10^7	4.3×10^5	1.46×10^5	8.44×10^4
Mean log reduction		1.60	2.07	2.31
Mean reduction (%)		97.5	99.2	99.5
Enterococcus faecalis				
Mean bacterial count (CFU/ml)	4.31×10^7	4.5×10^7	3.39×10^7	7.13×10^5
Mean log reduction		–0.02	0.10	1.78
Mean reduction (%)		–4.35	21.5	98.4

is composed of peptidoglycan, whereas the lipoprotein content is only about 3% [37].

Our observations show that Nd:YAG laser radiation is a powerful agent in reducing both gram-negative and gram-positive test organisms even through the dentin. Yet SEM findings and bacterial counts reveal an exceptionally different vulnerability of the strains.

One of the significant findings of our study was that *E. coli* showed striking perturbations of the cell envelope even at “subtherapeutic” settings. Although irradiation at 1.0 W, 15 pps had no effect on *E. faecalis*, the cell envelope of *E. coli* showed nodular structures and bleb formation.

This observed structural alteration is in accord with microbiologic findings identifying surface blebbing as the first morphologic change occurring in the permeability barrier of gram-negative bacteria under mild heat stress [38,39]. Katsuij et al. [40] suggested that blebbing results from the heat-induced release of membrane lipids and that the blebs consist of outer membrane ma-

terials. He deducted that the loss of the connection between the outer membrane and the underlying peptidoglycan induces this abnormality.

Studies of mutant strains with a genetic defect of these very lipoproteins revealed that the consequences of this structural abnormality resulted in the formation of outer membrane blebs and physiological deficiencies such as decreased growth rate and hypersensitivity to detergents [41,42]. Yet there is some disagreement among researchers as to whether or not this structural change is the direct cause of cell death.

This study presents morphologic evidence that the threshold energy inflicting structural changes upon gram-negative microorganisms in the deep layers of dentin is below the standard power settings recommended for the use of the Nd:YAG laser in clinical practice. Although the observed damage might not be immediately lethal, it is very likely to severely weaken the microorganism as a whole and to limit its long-term survivability in the inhospitable environment of an endodontically treated tooth. Our SEM investigations showed that visible injury patterns inflicted on *E. coli* increased dramatically with the application of 1.5 W, 15pps.

Irradiation of *E. faecalis* at the same settings yielded different results. One cycle of irradiation at 1.0 W, 15 pps or one cycle at 1.5 W, 15 pps neither caused striking degenerative alterations nor a decrease of colony forming units. Only two cycles of laser treatment led to substantial morphologic changes correlating to a reduction in bacterial counts. Although irradiation seems to have a strong membrane-disorganizing effect on *E. coli*, the cell wall of *E. faecalis* shows remarkable rigidity, which might be central to its persistence.

Furthermore, our observations suggest that the lethal effect of laser irradiation on microorganisms is based on a cumulative effect. This is in accordance with the mathematical model for cellular injury proposed by Jung [43]. A stress factor such as heat causes nonlethal reversible damage, which is then converted into lethal damage during repetition of the stress, a mechanism known as “knock-on effect.”

The findings reported here illustrate the extent and sequentiality of cell damage occurring during laser treatment of dentin at the standard therapeutical setting that has been proved to be safe for the surrounding anatomic structures. They underline that Nd:YAG laser irradiation is able to disinfect even deeper layers of contaminated dentin.

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